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Slit Antagonizes Netrin-1 Attractive Effects during the Migration of Inferior Olivary Neurons

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Inferior olivary neurons (ION) migrate circumferentially around the caudal rhombencephalon starting from the alar plate to locate ventrally close to the floor-plate, ipsilaterally to their site of proliferation. The floor-plate constitutes a source of diffusible factors. Among them, netrin-1 is implied in the survival and attraction of migrating ION *in vivo* and *in vitro*. We have looked for a possible involvement of slit-1/2 during ION migration. We report that: (1) *slit-1* and *slit-2* are coexpressed in the floor-plate of the rhombencephalon throughout ION development; (2) *robo-2*, a slit receptor, is expressed in migrating ION, in particular when they reach the vicinity of the floor-plate; (3) using *in vitro* assays in collagen matrix, netrin-1 exerts an attractive effect on ION leading processes and nuclei; (4) slit has a weak repulsive effect on ION axon outgrowth and no effect on migration by itself, but (5) when combined with netrin-1, it antagonizes part of or all of the effects of netrin-1 in a dose-dependent manner, inhibiting the attraction of axons and the migration of cell nuclei. Our results indicate that slit silences the attractive effects of netrin-1 and could participate in the correct ventral positioning of ION, stopping the migration when cell bodies reach the floor-plate. © 2002 Elsevier Science (USA)

Key Words: **nuclear translocation; axon outgrowth; brainstem; floor-plate; precerebellar nuclei; chemotropic molecules; robo receptors; collagen assays; *in situ* hybridization; mice.**

INTRODUCTION

Newly generated neurons need to move, sometimes for very long distances, from their proliferative zones to their final destinations. The molecular mechanisms that guide the migrating neurons have begun to be unraveled in recent years. Chemotropic molecules, in particular those expressed by the floor-plate, can provide either attractive or repulsive guidance cues; among them are members of the netrin (Serafini *et al.*, 1994) and slit protein families (Brose *et al.*, 1999). Both families of chemotropic cues are involved in axon guidance (Serafini *et al.*, 1994; Kennedy *et al.*, 1994; Brose *et al.*, 1999; Nguyen Ba-Charvet *et al.*, 1999) and neuronal migration (Bloch-Gallego *et al.*, 1999; Yee *et al.*, 1999; Hu, 1999; Wu *et al.*, 1999; Zhu *et al.*, 1999; Alcantara *et al.*, 2000; Hamasaki *et al.*, 2001). The effect of these diffusible molecules depends on the presence of different receptors and on the second messengers transducing the signal (Ming *et al.*, 1997; Song *et al.*, 1998; Bashaw and Goodman, 1999; Hong *et al.*, 1999). In addition, there is increasing evidence in favor of combined effects exerted by several chemotropic molecules operating simultaneously on the same guiding process (Zou *et al.*, 2000; Stein and Tessier-Lavigne, 2001).

The present study focuses on the inferior olivary neurons (ION), the precerebellar neurons that give rise to all climbing fibers of the cerebellum. ION originate in the most caudal region of the rhombic lips (Ellenberger *et al.*, 1969), located dorsally at the lateral edge of the rhombencephalon, and migrate ventrally by circumnavigating the ventrolateral aspect of the brainstem to settle in a ventral position. The neuron's leading process leads the way, imposing the migratory route until reaching and crossing the floor-plate (Bourrat and Sotelo, 1988). The ION nuclei translocate through the leading processes along the migratory stream but stop upon reaching the floor-plate, while the leading processes grow to the contralateral cerebellum (Altman and Bayer, 1987; Bourrat and Sotelo, 1988). The decision of the cell bodies to stop at the floor-plate is essential because it will determine the contralaterality of the final cerebellar projections. These developmental observations suggest that the floor-plate may act as an intermediate target, releasing signals that direct axon growth and migration of the ION (Bourrat and Sotelo, 1990).

We had previously shown that netrin-1 was involved in

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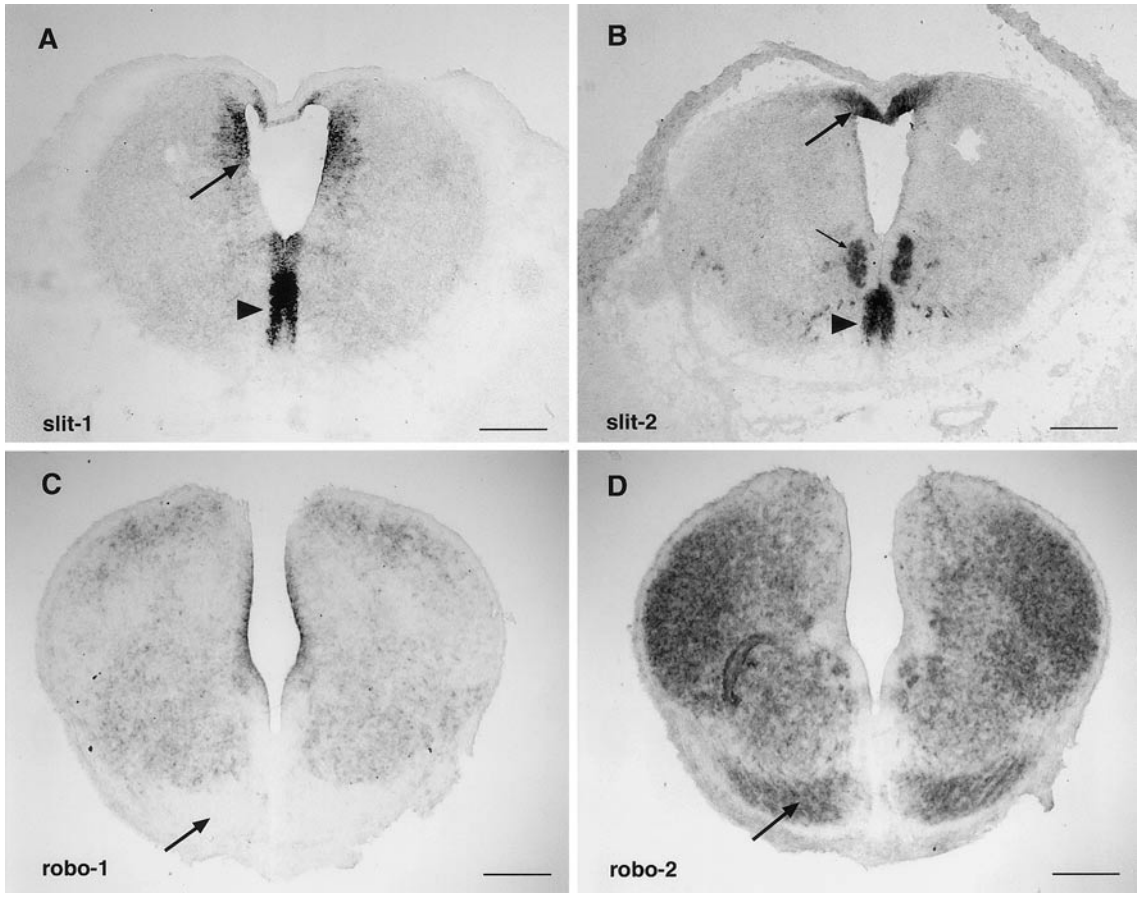

FIG. 1. Expression of *slit-1/slit-2* and *robo-1/robo-2* in wild-type mouse. At embryonic day 12, *slit-1* transcripts (A) are expressed in both floor-plate epithelial cells (arrowhead) and in cells located in the rhombic lips, dorsally to the *sulcus limitans* (arrow). *Slit-2* transcripts are expressed in the floor-plate cells (arrowhead), in the roof-plate (arrow), and in motoneurons in the hypoglossal nucleus (thin arrow). At embryonic day 13 (E13), *robo-1* transcripts (C) are totally absent from the ION region (arrow), whereas *robo-2* is widely expressed in the caudal brainstem, specially by IONs that reach the vicinity of the floor-plate (arrow). Scale bar: (A, B) 160 μm; (C, D) 200 μm.

the migration of ION (Bloch-Gallego *et al.*, 1999). In mice deficient for the expression of netrin-1, most ION fail to reach the floor-plate and are located ectopically in the migratory stream, suggesting a lack of ION attraction. Recent data obtained from *in vitro* grafts of ectopic floor-plates (de Diego *et al.*, 2002) report that the floor-plate also exhibited a short-range stop signal for migrating ION, but the molecular nature of this signal was not identified. Since the family of slit proteins prevents midline crossing in several axon guidance systems (Bagri *et al.*, 2002; Plump *et al.*, 2002), we tested its possible involvement in controlling ION migration. We have first established the expression pattern of *slits* and of their receptors *robo* throughout the ION development in mice. Using *in vitro* confrontation assays in collagen matrix, we have analyzed the effects of various sources of netrin-1 and slit—on ION axon guidance and nuclei migration—when applied alone or in combination. We report here that netrin-1 has attractive effects on both axons and nuclei, and that nuclear migration is a netrin-1 dependent process; slit can silence netrin-1 effects and could be involved at a point of the migratory process, to stop nuclear translocation and allow the correct ventral positioning of the migrating ION.

MATERIALS AND METHODS

Fixation

Mouse embryos (E11–E13) were obtained from timed matings of outbreed Swiss mice (Janvier, Le Genest St Isle, France). They were fixed by immersion in 4% paraformaldehyde (PF) in phosphate buffer, pH 7.4, for 1 h. The neural tubes were dissected out, cryoprotected overnight with 10% sucrose solution (in phosphate buffer, pH 7.4), and embedded in 7.5% gelatin/10% sucrose. Then, they were frozen in isopentane cooled to −55°C with liquid nitrogen and serially sectioned in a cryostat in the frontal plane. The sections (20-μm thick) were mounted on four parallel sets of slides.

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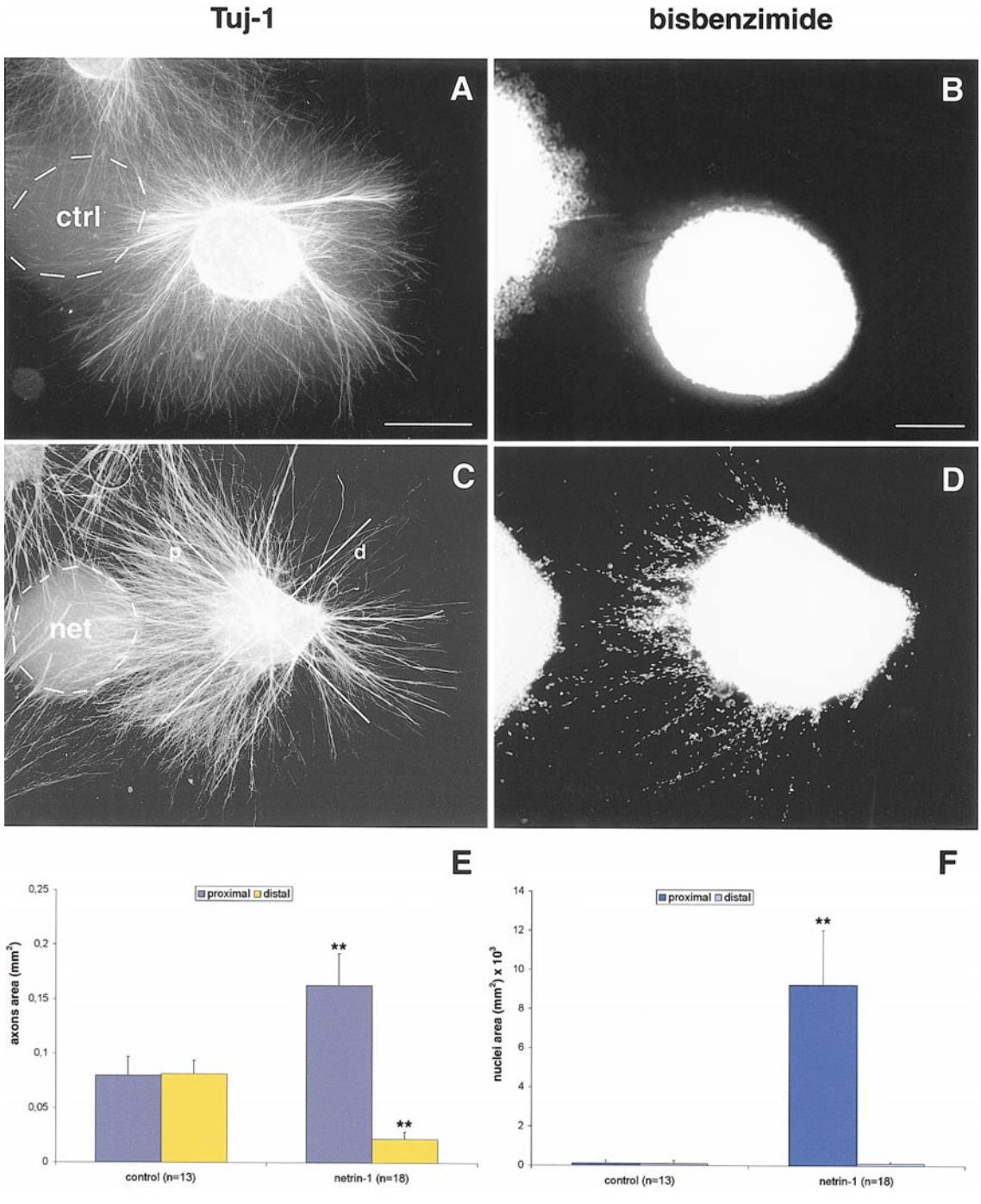

FIG. 2. Netrin-1 is strictly required for chemoattraction and nuclear translocation. Axon outgrowth and neuronal migration (nuclear translocation) were analyzed in confrontation assays after 3 DIV. The explants were immunostained with class III β-tubulin antibodies Tuj-1 (left column), and the cell nuclei of the same preparations—illustrated at a higher magnification—were visualized with bis-benzimide (right column). When ION explants are faced with mock EBNA cells (surrounded with the dotted line in A), neurite outgrowth develops all around the explants (A) and no nuclear translocation occurs (B). On the contrary, when ION explants are faced with netrin-1-secreting EBNA cells (surrounded with the dotted line in C), both neurite outgrowth (C) and nuclear translocation (D) develop and occur mainly in proximal quadrant (p) compared with distal quadrant (d) as defined in (C). Histograms (E) and (F) illustrate the quantification and statistical analysis of axon outgrowth (E) and cell migration (F) of ION explants. Histogram (E) illustrates the surface covered by neuritic processes in the proximal and distal quadrants of ION explants cocultured and faced with EBNA-mock cells or netrin-1 EBNA-secreting cells. **, $P < 0.001$, compared with corresponding quadrants in control. Histogram (F) represents the surface covered by cell nuclei in both proximal and distal quadrants in the same various conditions as in (E). **, $P < 0.001$, compared with corresponding quadrants in control. Scale bar: (A, C) 500 μm; (B, D) 200 μm.

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Probes for in Situ Hybridization

In situ hybridization (ISH) was performed on cryosections. Antisense and sense probes were respectively obtained for: a rat *slit-1* probe (Brose *et al.*, 1999) linearized with *Bam*HI or *Hin*dIII (Pharmacia) and transcribed in the presence of digoxigenin (Dig)-UTP (Boehringer Mannheim) using T7 RNA polymerase or T3 RNA polymerase (Riboprobe Kit; Promega) to produce the antisense or sense probes; a rat *slit-2* probe (Brose *et al.*, 1999) linearized with *Xba*I or *Kpn*I and transcribed in the presence of Dig-UTP using T7 RNA polymerase or T3 RNA polymerase to produce the antisense or sense probes; a rat *robo-1* (Brose *et al.*, 1999) was linearized with *Xba*I or *Xho*I and transcribed in the presence of Dig-UTP using T3 or T7 RNA polymerase to produce respectively the antisense or sense probes; a rat *robo-2* (Brose *et al.*, 1999) linearized with *Not*I or *Xho*I and transcribed in the presence of Dig-UTP using T7 or T3 RNA polymerases to produce the antisense or sense probes; a mouse *Brn-3.b* probe (Wyatt *et al.*, 1998) linearized with *Not*I or *Nco*I, and transcribed in the presence of Dig-UTP using T7 or SP6 RNA polymerase to produce respectively the antisense or sense probes. ISH was carried out according to Myat *et al.* (1996); the probes were revealed using an anti-digoxigenin-alkaline-phosphatase Fab antibody (1:2000; Boehringer Mannheim) and NBT-BCIP (Boehringer, Mannheim), as a substrate for the alkaline phosphatase. No signal was obtained when using the sense probes.

Explant Cultures and Cocultures

The *medulla oblongata* from E11 Swiss mice embryos were dissected. Using a Tissue chopper, 300-μm-thick coronal sections were performed and collected in GBSS (Life Technologies) supplemented with 0.5% glucose. Their most dorsal portions, which include the rhombic lips and the alar subventricular zone which is largely enriched in young postmitotic ION, as well as young ION initiating their migration in the most dorsal part of the submarginal stream and that express *Brn-3.b* transcripts (see Fig. 3A), were dissected out from those slices. We refer to this region as ION explants. At this stage, among neurons proliferating in the caudal rhombic lips, only IONs have achieved their last division (Taber-Pierce, 1973). ION explants were collected and incubated until all embryos had been assembled in the culture medium, which consists of DMEM (Life Technologies) supplemented with L-glutamine (2 mM), 5% horse serum, 5% fetal calf serum, penicillin/streptomycin (100 UI/ml), and N2 and B27 supplements (all from Life Technologies). Explants were cocultured with aggregates obtained from hanging drops (as previously described by Kennedy *et al.*, 1994) of mock EBNA 293 cell line (Invitrogen), EBNA 293 secreting chick netrin-1 (EBNA-net-1; Shirasaki *et al.*, 1996), *Xenopus* slit (EBNA-slit; Wu *et al.*, 1999), or COS cells transfected with *Xenopus slit* or mouse *slit-1* fused in frame at its C terminus to a myc tag (COS-slit; Li *et al.*, 1999). The *Xenopus slit* is an ortholog of the mouse and human *slit-2* genes (Li *et al.*, 1999). In some experiments, ION explants were cultured in the presence of conditioned medium from mock EBNA cells, or from EBNA cells secreting netrin-1 that had been maintained for 6–7 days *in vitro* (DIV), and mixed v/v with the culture medium. All explants were embedded in rat tail collagen gel as previously described (Lumsden and Davies, 1986) and cultured for 60–64 h in a 5% CO_2, 37°C, 95% humidity incubator. After each experiment, expressions of slit and/or netrin-1 were controlled on Western blots by using the monoclonal 9E10 anti-myc antibody (maintained by the University of Iowa, IA City, Hybridoma Bank, DSHB; Evan *et al.*, 1985). We quantified on Western blots (using Metamorph analysis program) the amount of slit protein secreted from a 25-μl drop of EBNA-slit with the amount of slit protein secreted from a 25-μl drop of COS-slit obtained from the similar amounts of seeded cells 1 day prior to doing the drops.

For the visualization of neuronal processes, cultures were fixed for 1 h in 4% PF, rinsed several times, incubated with a neuron-specific anti-class III β-tubulin monoclonal antibody (Tuj-1, 1:2000; Jackson Immunological Laboratories), and revealed with a Cy3-conjugated goat anti-mouse IgG antibody (1:200; Amersham). Cell nuclei in explants and migrating cells were visualized with bis-benzimide (1 μg/ml, 30 min incubation at room temperature; Vector), rinsed several times, and mounted in Crystal Mount (Biomedia, EMS). For the visualization of transcripts in collagen assays, cultures were fixed for 1 h in 4% PF, rinsed several times, cryoprotected, embedded, and frozen as described above in the section "fixation," and serially sectioned (20-μm thick) in a cryostat in the horizontal plane.

Scoring of Cell Migration and Quantitation of Leading Process Outgrowth and Cell Migration

Microphotographs of each collagen assay were acquired from a Leica fluorescent microscope through a Photometrics coolscan monochrome CCD camera from Roper Scientific Photometrics by using Metamorph area imaging. Axon outgrowth was first obtained with Cy3 filters to visualize Tuj-1 immunostained fibers. Thereafter, the visualization of nuclear cell migration was carried out by illuminating the explants with filters to observe bis-benzimide staining. Each assay was divided in four quadrants, and the orientation and distribution of both neurites and cell migration were analyzed and scored as follows. For each explant, the areas covered by either Tuj-1-positive neurites or by bis-benzimide-stained nuclei outside the explant were measured in both proximal and distal quadrants after threshold for light objects, using the Metamorph area analysis program (Princeton Instruments).

To obtain a quantitative evaluation of the chemotropic effects, the area covered by axons in the proximal quadrant was divided by the corresponding area in the distal quadrant for each explant. When the proximal/distal ratio (P/D ratio) is close to 1, there is no chemotropic effect. A ratio higher than 1 defines an attraction, whereas a ratio lower than 1 defines a repulsion.

The mean areas and standard deviations (SD) were calculated. Data were statistically analyzed by using the Student's *t* test and the nonparametric Mann–Whitney test. Differences were considered as significant when $P < 0.001$ using the Student's *t* test or when $P < 0.01$ using both Student's *t* and Mann–Whitney tests.

RESULTS

Expression of slit-1/2 and robo-1/2 during the Migration of IONs in Mice

At E12, coincident with the initiation of the ION migration, *slit-1* (Fig. 1A), *slit-2* (Fig. 1B), and *netrin-1* (Bloch-Gallego *et al.*, 1999; not illustrated) transcripts were highly expressed by the rhombencephalic floor-plate epithelial cells. In addition, *slit-1* was highly expressed dorsally to the *sulcus limitans* and in the neuroepithelial cells of the alar plate (Fig. 1A). *Slit-2* transcripts (Fig. 1B) were expressed in the roof-plate, while *netrin-1* was expressed in the basal

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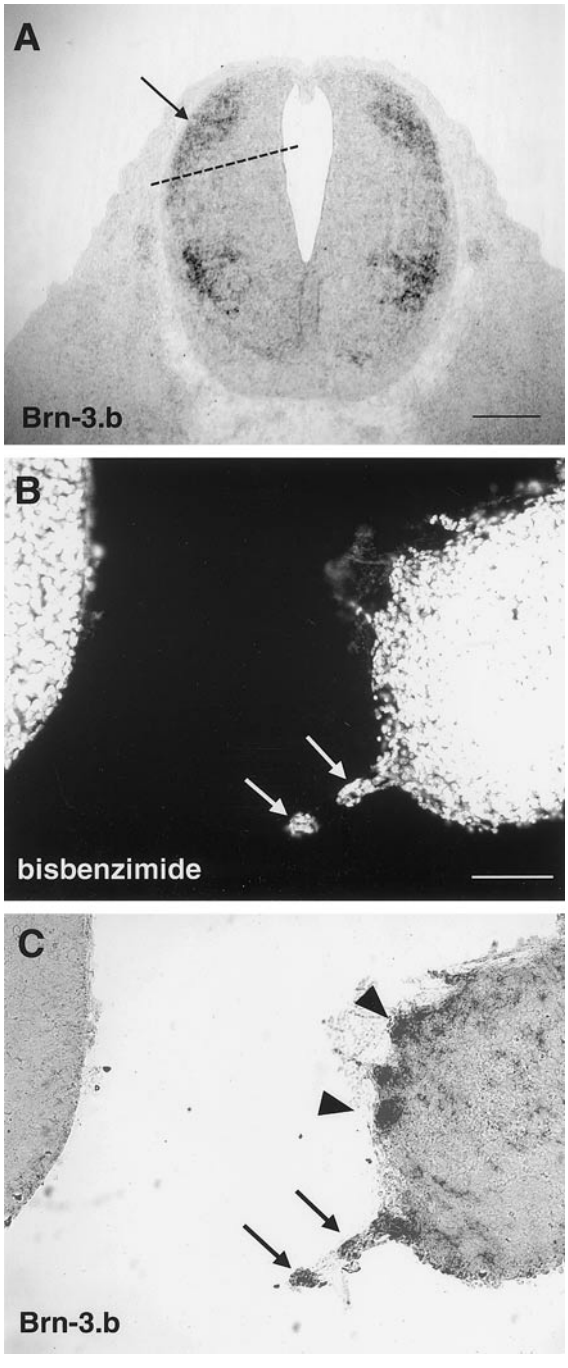

FIG. 3. Expression of *Brn-3.b* transcripts in ION explants and in neurons that migrate toward netrin-1-secreting cells. At E11.5, *Brn-3.b* transcripts are expressed by IONs that are located in the dorsal part of the submarginal stream, once they have left the rhombic lips (arrow). The most dorsal portion of the section (delimited with the dotted line) is used as ION explants in collagen assays. After 3 DIV, neurons that migrate out of the explants toward netrin-1-secreting cells are visualized with bis-benzimide (B) on a cryosection of the collagen assay, and all of them express *Brn-3.b* transcripts (arrows in C). Neurons that express *Brn-3.b* transcripts are also located at the periphery of the explants (arrowheads in C). Scale bar: (A) 160 μm; (B, C) 100 μm.

plate. At E13, *robo-1* transcripts (Fig. 1C) were weakly but extensively expressed in the brainstem, but they were completely absent from the ION medioventral region at E13 (arrow; Fig. 1C); *robo-2* expression (Fig. 1D) was widespread in the brainstem, including the spinal trigeminal nucleus, and the IONs when they reach the midline (arrow in Fig. 1D) at an advanced stage of their migration process. IONs were identified by their expression of *Brn-3.b* transcripts on alternate sections (not illustrated; Turner *et al.*, 1994; Bloch-Gallego *et al.*, 1999). *Robo-2* expression remained in adulthood (not illustrated; see Marillat *et al.*, 2002 for expression in rat). Thus, the expression pattern of *slit-1*, *slit-2*, and *robo-2* is of special interest for investigating cues that direct ION migration and cues that stop the migration.

Netrin-1 Attracts ION Axons and Is Absolutely Required for ION Migration

To characterize the effect of netrin-1 on ION migration, we used the *in vitro* confrontation assay by facing explants containing young postmitotic ION (ION explants; see Fig. 3A) with netrin-1-secreting EBNA cells in a collagen matrix. Both neurite outgrowth and neuronal migration were analyzed after 3 DIV. When ION explants were faced with mock EBNA cells, there was a symmetric outgrowth of neurites all around the explants (Figs. 2A and 2E; and Table 1), but almost no nuclear translocation (Figs. 2B and 2F; Table 1). In contrast, explants facing netrin-1-secreting cells had an asymmetric pattern of neurite outgrowth with growth mostly in the proximal quadrant (i.e., facing the source of netrin-1). This asymmetry is reflected by a P/D ratio of 7.8 $\pm$ 1.7 (Figs. 2C and 2E; and Table 1). The process of nuclear translocation paralleled this oriented axon outgrowth: numerous nuclei left the explants in the proximal quadrant and formed long chains of migrating cells that are oriented toward the source of netrin-1 (Figs. 2D and 2F). These *in vitro* assays demonstrate that netrin-1 has a direct chemoattractant effect on axon outgrowth and a permissive and attractive action on the migration of ION.

The bis-benzimide-labeled neurons that left the explants toward a netrin-1 source (Fig. 3B) expressed *Brn-3.b* transcripts (Fig. 3C), confirming their ION identity (Turner *et al.*, 1994). Moreover, the migrating cell nuclei localized within the neuronal leading processes (Fig. 4A–4C) that formed Tuj-1-positive fascicles. These are characteristic features of ION neurophilic migration.

Slit As a Functional Partner of Netrin-1 during ION Migration: Silencing of Netrin-1-Mediated Effects on Axon Outgrowth and Neuronal Migration

Slits are known as repulsive molecules in various neuronal migratory systems (Hu 1999; Wu *et al.*, 1999; Zhu *et al.*,

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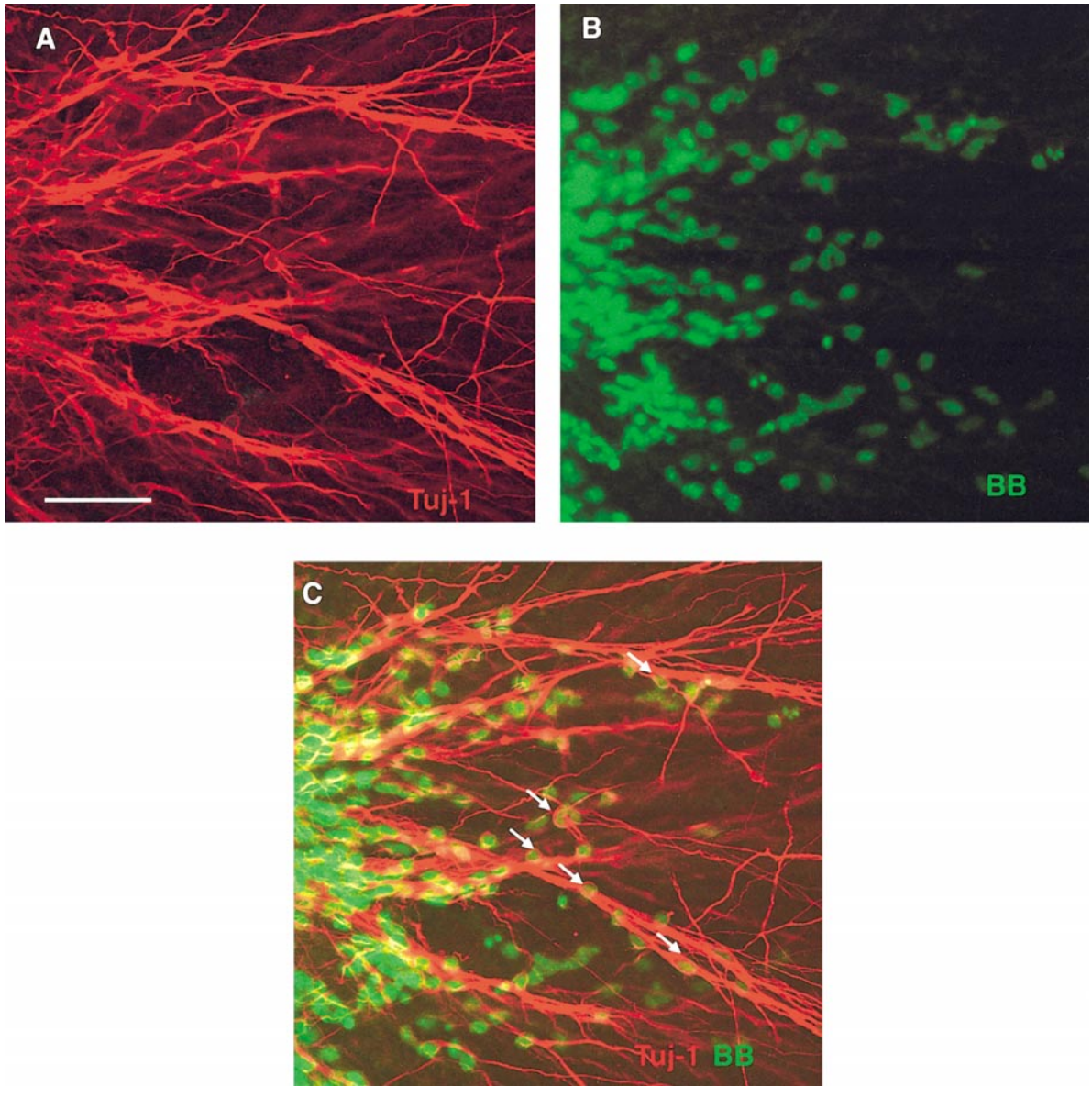

FIG. 4. High magnification of nuclear translocation in the leading processes. Axons were immunostained (A) with class III β-tubulin antibodies Tuj-1, and nuclei were stained with bis-benzimide (B). Images were then acquired by using a Leica Microsystem confocal microscope (SP2). Overlays (C) were prepared by using Photoshop 6.0 (Adobe) running on a G4 Macintosh (Apple). The picture illustrates a single section of 0.8-μm thickness. Cell bodies are surrounded by Tuj-1 labeling during their translocation (arrows in C). Scale bar, 50 μm.

FIG. 5. Slit-secreting cells antagonize both axon outgrowth and nuclear migration permissive effects of netrin-1-conditioned medium. Axon outgrowth and neuronal migration (nuclear translocation) were analyzed in confrontation assays after 3 DIV. The explants were immunostained with class III β-tubulin antibodies Tuj-1 (left column), and the cell nuclei of the same preparations were visualized with bis-benzimide (right column) at a higher magnification. When ION explants are faced with the slit-secreting cells alone, axon outgrowth develops preferentially in the lateral and distal quadrants, whereas weakly in the proximal quadrant (A), and no migration occurs in any direction (B). When ION explants are faced with mock-EBNA cells in the presence of soluble netrin-1 (conditioned medium), axon outgrowth (C) and nuclear migration (D) strongly develop all around the explant in any direction. When faced with slit EBNA-secreting cells, both axon outgrowth (E) and nuclear migration (F) are reduced twice in the proximal quadrant, and the aspect of neurites differs in both proximal and distal quadrants. Histograms (G) and (H) illustrate the quantification and statistical analysis of axon outgrowth and cell migration of ION explants. Histogram (G) illustrates the surface covered by neuritic processes in the proximal and distal quadrants of ION explants faced with either EBNA-mock cells or EBNA-slit cells, in the presence of soluble netrin-1. **, $P < 0.001$ compared with proximal outgrowth in explants faced with control cells. Histogram (H) represents the surface covered by cell nuclei in both proximal and distal quadrants in the same various conditions as in (E). **, $P < 0.001$ compared with proximal quadrant in explants faced with control cells. Scale bar: (A, C, E) 500 μm; (B, D, F) 200 μm.

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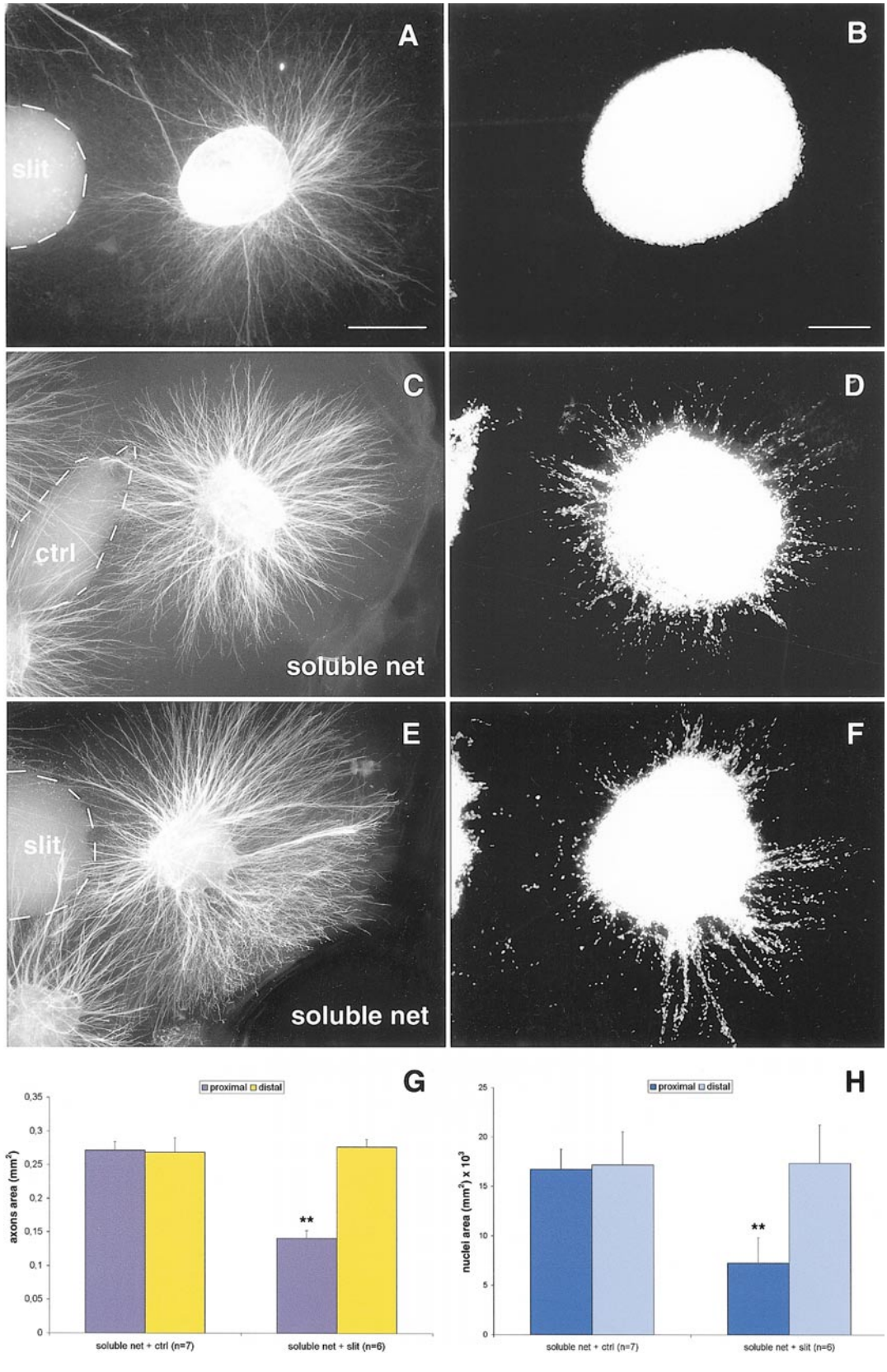

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TABLE 1

	Axon outgrowth			Nuclei migration		
	mean area (mm^2) ± SD			mean area (mm^2) ± SD × 10^3		
	proximal	distal	P/D ratio	proximal	distal	*n*
Control	0.080 ± 0.017	0.081 ± 0.012	1.0 ± 0.2	0.1 ± 0.2	0.1 ± 0.2	13
Netrin-1	0.163 ± 0.029	0.022 ± 0.006	7.8 ± 1.7	9.2 ± 2.8	0.1 ± 0.1	18
Low slit + net	0.190 ± 0.047	0.062 ± 0.017	3.3 ± 1.0	13.2 ± 6.1	3.4 ± 3.1	7
High slit + net	0.081 ± 0.013	0.056 ± 0.014	1.5 ± 0.4	0.5 ± 0.5	0.4 ± 0.4	16
Slit	0.058 ± 0.014	0.086 ± 0.026	0.7 ± 0.2	0.1 ± 0.1	0.2 ± 0.1	13
Soluble net + ctrl	0.272 ± 0.012	0.269 ± 0.021	1.0 ± 0.1	16.7 ± 2.1	17.2 ± 3.4	7
Soluble net + slit	0.141 ± 0.011	0.276 ± 0.011	0.5 ± 0.0	7.3 ± 2.6	17.4 ± 3.9	6

Note. The areas occupied by neuritic processes or by cell bodies in ION explants cocultured with aggregates of mock or netrin-1/slit-secreting cells in various combinations were measured and expressed as means ± SD.

1999). To determine whether slit is involved in ION migration, we tested the effect of slit, alone or in combination with netrin-1, in the *in vitro* confrontation assays. When ION explants faced EBNA-slit cells, no nuclear translocation was visible after 3 DIV and the cell nuclei remained within the explants (Fig. 5B). Axon outgrowth developed preferentially in the lateral and distal quadrants rather than in the proximal one (Table 1; Fig. 5A). Thus, slit had no effect on nuclear migration but had a small repulsive effect on axon outgrowth.

The results were completely different when the combined action of slit and netrin-1 were tested. In a first set of experiments, ION explants were confronted with EBNA-mock cells or EBNA-slit cells, in the presence of netrin-1-conditioned medium (i.e., soluble netrin-1). With EBNA-mock cells (Figs. 5C and 5D), axon outgrowth and nuclear migration occurred in all directions around the ION explants (Table 1; Figs. 5G and 5H). When faced with EBNA-slit cells in the presence of soluble netrin-1 (Figs. 5E and 5F), there was a twofold reduction of axon growth in the proximal quadrant as compared with the control conditions (EBNA-mock cells). Furthermore, the rate of nuclear migration was reduced by 50% in the proximal quadrant (Fig. 5H). In contrast, the surface covered by axons and by migrating nuclei remained unchanged in the distal quadrant (Table 1; Figs. 5E–5H). In addition, qualitative morphological differences were observed, in that the axons growing toward the netrin-1 source were more fasciculated than those growing in the opposite direction (Fig. 5E).

A second set of experiments consisted of facing ION explants with a local source of netrin-1/slit combination and varying slit concentrations in this mixed source. The approximate amount of secreted slit or netrin-1 from the transfected cells was evaluated in Western blots. As illustrated in Fig. 6H, EBNA-slit cells secreted about 10 times more slit than transiently transfected COS-slit cells (all three forms of slit, either cleaved or not, were taken into account). The hanging drop approach was used in which either one-third of COS-slit cells and two-thirds of EBNA-netrin-1 cells (slit/netrin-1 = 1/3 referred to "low slit" in Fig. 6) or EBNA-slit cells and EBNA-netrin-1 cells at 1:1 ratio (slit/netrin-1 = 1/1 referred to "high slit" in Fig. 6) were mixed. Control experiments were performed by mixing netrin-1-secreting cells with mock-COS or EBNA cells. In the confrontation assays, the addition of slit dramatically changed the action of netrin-1, and these changes were dose-dependent. When low doses of slit were added to netrin-1, the chemoattractant effect of netrin-1 was decreased but not suppressed (Fig. 6A): the surface covered by outgrowing axons did not change in the proximal quadrant but increased in the distal quadrant, resulting in a decreased P/D ratio of 3.3 ± 1.0 (Table 1; compare Fig. 6A and Fig. 2C; Figs. 6E–6G). Moreover, migration was no longer restricted to the proximal quadrant, and bis-benzimide nuclear staining revealed chains of migrating neurons along the axons fascicles in the proximal, lateral, and even distal quadrants in some cases (Figs. 6B and 6F). These results show that the addition of low concentrations of slit to the netrin-1 gradient does not significantly modify the permissive action of netrin-1 ($P = 0.044$ using Student's t test, but $P > 0.1$ using Mann–Whitney test) on the initiation of axon outgrowth and migration, but specifically decreased the directionality of the chemoattractive effect of netrin-1 on axon outgrowth and neuronal migration.

In confrontation assays with higher concentrations of slit (high slit + netrin-1), the attractive effects of netrin-1 on ION axons were severely reduced but not completely suppressed (P/D of 1.5 ± 0.5, Table 1; Figs. 5C and 5G). Moreover, nuclear translocation was drastically affected and most cell nuclei remained within the explants (Table 1; Figs. 5D and 5F). These observations suggest that high concentrations of slit inhibit the permissive action of netrin-1 on the migration of postmitotic ION, in addition to strongly silencing its chemoattractive effects on axon outgrowth. We conclude that slit can antagonize the attractive

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effects of netrin-1 on ION axon guidance and cell migration in a dose-dependent manner.

DISCUSSION

Netrin-1 has previously been shown to be a key factor for the migration of ION, possibly as a guidance cue for the migration from the rhombic lips (Bloch-Gallego *et al.*, 1999). Here, we show that, *in vitro*, netrin-1 has permissive migratory effects and also acts as a chemoattractant for directing axon outgrowth and nuclear migration. We provide a detailed cartography of *slit*/*robo* expression consistent with a physiological involvement of netrin-1 and slit during the tangential migration of ION. The combined effects of netrin-1 and slit revealed that slit exerts an antagonistic effect on netrin-1: at low doses, it diminishes the chemoattractant effect of netrin-1 on axon outgrowth and nuclear migration; at higher doses, slit antagonizes both migratory permissive effects of netrin-1 and most of its guidance effects.

Netrin-1 Directs Axon Outgrowth and Allows Migration of ION

The ION migration consists of nuclear translocation within its own preexisting axon. Consequently, the cues that orient axon outgrowth are appropriate candidates to provide directionality to the tangentially migrating cell bodies. We report here that netrin-1 attracts both axons and cell bodies. However, in the absence of netrin-1, no migration occurs, whereas axon outgrowth develops. Netrin-1 is an attractive factor for both ION axons and cell bodies, and is strictly required for migration to occur *in vitro*. This is in agreement with the analysis of netrin-1 mutant mice in which the ION cell bodies are located ectopically along the migratory stream, suggesting a lack of ION attraction by the floor-plate (Bloch-Gallego *et al.*, 1999). In agreement with previous studies on later generated precerebellar neurons, including those belonging to the pontine basilar and lateral reticular nuclei (Yee *et al.*, 1999; Alcantara *et al.*, 2000), we conclude that netrin-1 orients the migration of various populations of precerebellar neurons.

Slit Repels Axons and Prevents Netrin-1 Permissive Effects for ION Migration

Possible involvement in the initiation of ION migration. We found that when ION explants were faced with slit, their axons were slightly but significantly repulsed from slit source and migration did not occur. However, when ION explants were faced with slit-secreting cells in the presence of soluble netrin-1 (a condition where migratory permissive effects are enhanced), slit silenced the migratory permissive effects of netrin-1 in the proximal quadrant, inhibiting nuclear migration toward the source of slit. Thus, a local source of slit diminishes ION axon outgrowth and prevents nuclear translocation. *In vivo*, *slit-1* is expressed in the alar plate of the ventricular zone (that contains precursors of ION), apposed with *netrin-1*-expressing cells of the basal plate and *slit-2* in the roof-plate. The presence of slit sources in the dorsal regions of the rhombencephalon could allow the leading processes of the ION to leave their proliferative zone and grow circumferentially toward the ventral aspect of the brainstem. Even though slit alone is not able to repel ION cell nuclei, the presence of netrin-1 in the basal plate and the floor-plate could account for the permissivity of the migration.

Slit as a presumptive cofactor of netrin-1 for stopping ION nuclear translocation. It has been recently shown from *in vitro* assays (de Diego *et al.*, 2002), that the floor-plate exhibits a short-range stop signal for migrating ION. In the present work, we report that slit antagonizes the effects of netrin-1 in a dose-dependent manner: at low doses, it antagonizes only the attractive effect of netrin-1 on ION axons, whereas at higher doses, slit blocks both chemoattractive and migratory permissive effects of netrin-1. When IONs reach the floor-plate, the combination of a high concentration of slit with netrin-1 could inhibit the permissive migratory effects of netrin-1, and thus participate to stopping the migration of ION on both sides of the floor-plate. This process implies that slit has a low diffusion from its site of production, whereas netrin could diffuse more broadly, creating a larger chemotactic gradient than slit. This is supported by biochemical evidence, which indicates that slit interacts with cell membranes and can bind to extracellular matrix components (Brose *et al.*, 1999; Nguyen Ba-Charvet *et al.*, 2001). This would result in a netrin-slit antagonism only around the floor-plate.

How Do ION Axons Cross the Floor-Plate while Cell Bodies Are Stopped?

The behavior of migrating ION cell bodies is strikingly different from that of their growing axons when reaching the floor-plate. While the leading processes and the cell bodies are similarly attracted by netrin-1 during the initial steps of migration, they respond differently to a slit/netrin-1 combination at the floor-plate since axons but not cell bodies cross it. Similarly, in confrontation assays, when ION explants are faced with cells secreting high amounts of slit and netrin-1 (high slit + netrin-1), almost no nuclear translocation occurs, while the axons remain slightly but significantly attracted. Thus, slit does not completely antagonize netrin-1-attractive effects on axons, while it is a powerful inhibitor of nuclear migration. This could result from an asymmetric localization of intracellular molecules involved in netrin-1 and slit signaling cascades (including the membrane receptors and the second messengers) in the somatic and axonal compartments. Such a mechanism has been demonstrated for cortical pyramidal neurons that exhibit an asymmetric localization of soluble guanylate

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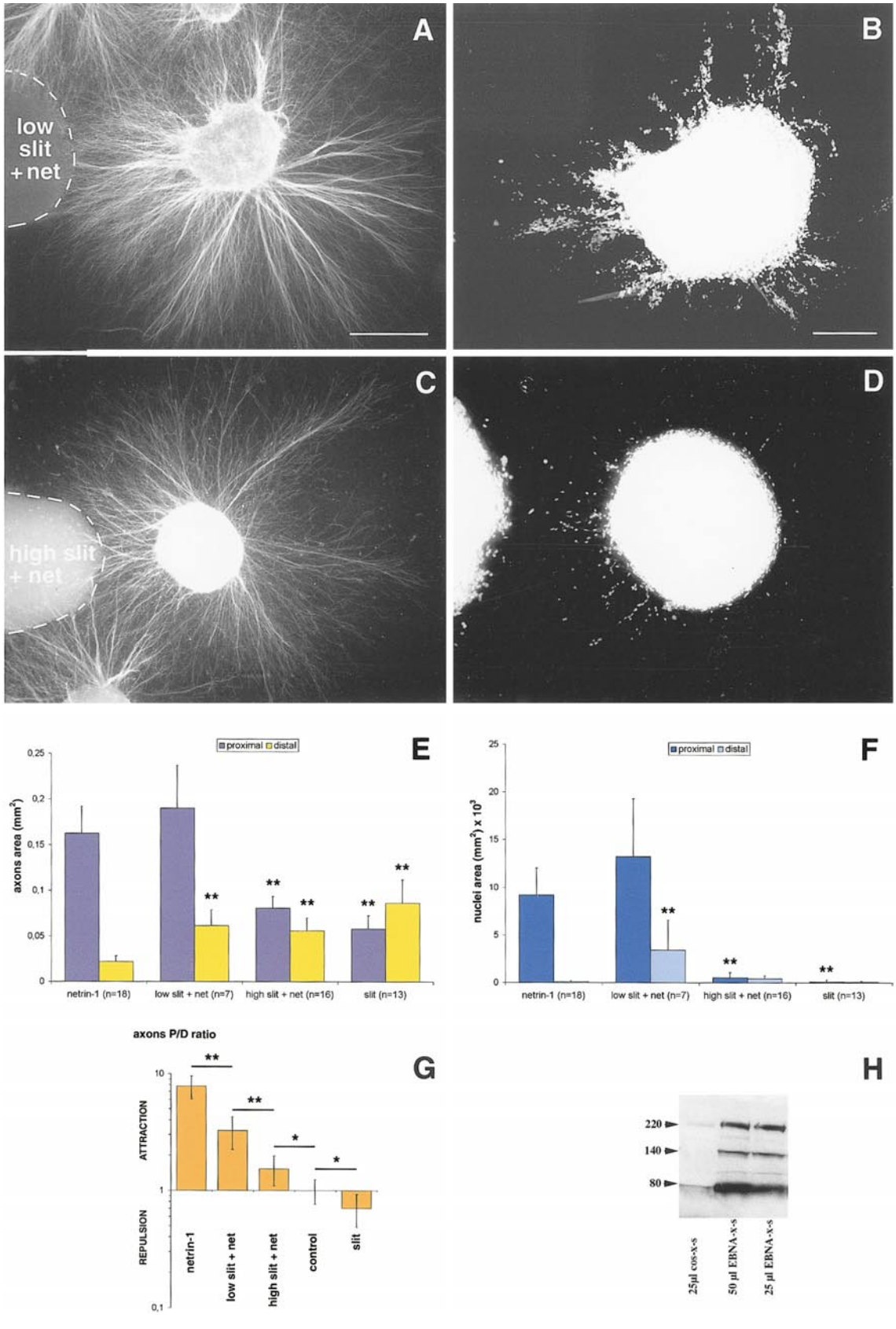

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cyclase which confers distinct Sema3A responses to axons and dendrites (Polleux *et al.*, 2000).

Crossing and Noncrossing Precerebellar Neurons Differently Express Robo-2 Receptor during their Migration

It is important to underline that *robo-1/2* transcripts are not expressed in neurons that form the lateral reticular and external cuneatus nuclei (Marillat *et al.*, 2002), whose cell bodies cross the floor-plate to develop an ipsilateral cerebellar projection. Robo-2 signaling could thus participate in sorting floor-plate crossing vs noncrossing precerebellar migratory neurons: the IONs which express *robo-2* do not cross the floor-plate likely due to the silencing action of slit, whereas the neurons that will form the lateral reticular and external cuneatus nuclei would lack *robo-2* expression and consequently could cross the floor-plate.

Possible Mechanisms for Slit Silencing of Netrin-1 Effects during ION Migration

We show that slit can silence the attractive effects of netrin-1 during the migration of ION. However, the molecular mechanisms of such an antagonization remain to be established. Stein and Tessier-Lavigne (2001) have described a similar silencing for axon guidance of spinal neurons from *Xenopus*. They showed that upon binding to slit, robo could interact with the netrin-1 receptor DCC (through their intracellular domains) and silence netrin-1-attractive effects. Such a complex interaction could also account for netrin-1 silencing by slit during the migration of ION since they express both *DCC* (Bloch-Gallego *et al.*, 1999) and *robo-2* when they reach the floor-plate. At E13, the highest levels of *DCC* transcripts were located in the subventricular zone of the alar plate, which contains postmitotic/premigratory precerebellar neurons, and in IONs located at both sides of the floor-plate (Bloch-Gallego *et al.*, 1999). But we cannot rule out the possibility that the silencing could result from two antagonist signaling pathways, a netrin-1/DCC pathway permissive for nuclear translocation and a slit/robo-2 pathway that inhibits nuclear translocation. Another possibility is that there is a direct interaction between netrin-1 and slit members: Brose *et al.* (1999) have reported that slit-2 could bind netrin-1 with a similar affinity as netrin-1 for its DCC receptor. However, it remains unclear whether such an interaction could decrease netrin-1 activity. These hypotheses still need to be tested through biochemical experiments.

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FIG. 6. Slit inhibits part or all of netrin-1 effects according to its concentration. The explants were immunostained with class III β-tubulin antibodies (A, C), and the cell nuclei of the same preparations were visualized with bis-benzimide (B, D) and illustrated at a higher magnification. Secreting cells are indicated by a dotted line. When ION explants taken from wild type caudal brainstem are faced with mixed cell aggregates secreting netrin-1 and a low slit concentration, both axon outgrowth (A) and cell migration (B) develop, but the orientation of both events is not strictly restricted to the proximal quadrant (B). When netrin-1-secreting cells are mixed with a 5- to 10-fold higher concentration of slit, axon outgrowth (C) develops bidirectionally in any quadrant and very few migrations can occur (D). (E–G) Quantification and statistical analysis of axon outgrowth (E and G), and cell migration (F) of ION explants cocultured faced with cell aggregates secreting various amounts of slit and/or netrin-1. (E) Axon outgrowth in distal and proximal quadrants was compared with axon outgrowth in presence of netrin-1. **, $P < 0.001$ compared with corresponding quadrants in ION explants faced with netrin-1-secreting cells. (F) The surface covered by migrating nuclei was compared with migration in ION explants faced with netrin-1-secreting cells in both distal and proximal quadrants. **, $P < 0.001$ compared with corresponding quadrants in ION explants faced with netrin-1-secreting cells. (G) P/D ratio of axon outgrowth: to obtain a quantitative evaluation of the chemotropic effects, the areas covered by axons in the proximal quadrants were divided by the corresponding areas in the distal quadrants. Logarithmic scale: P/D ratios were compared by pairs, *, $P < 0.01$ using the Student's t test and $P < 0.01$ using the nonparametric Mann–Whitney test; **, $P < 0.001$ using the Student's t test. (H) Western blot illustrating the different rates of slit secreted by COS transfected cells compared with the stable EBNA slit-secreting cell line. Scale bar: (A, C) 500 μm; (B, D) 200 μm.

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